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RESEARCH ARTICLE

Fungal inhibition by lactic acid bacteria (LAB) is modulated *in vitro* by cocoa fermentation-related conditions: towards a biocontrol of fungi in processing cocoa

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ABSTRACT

Fungal growth impair strongly the quality of processed cocoa, leading to low crops value for farmers and manufacturers. The antifungal activity of lactic acid bacteria (LAB) from fermenting cocoa was analyzed and the conditions of fungal inhibition were evaluated. Fungi were isolated from stored fermented and dried cocoa beans on sabouraud plate medium. The results showed that, LAB strongly inhibit fungi isolated from fermented cocoa beans notably Mucors and Aspergilli, with *Lactobacillus plantarum* inducing the stronger inhibition whereas *Leuconostoc mesenteroides* produced a weaker inhibition. Acids production assayed by HPLC was found to be not related to antifungal activity, since LAB strains producing strong antifungal activity were not necessarly the best acid producers. Maximum fungal inhibition occured at 35 °C in *Lactobacillus plantarum* and *Leuconostoc mesenteroides* but also at 30 °C in *Lactobacllus casei*. Likewise, a narrow acidic pH range (3.0-4.0) allowed full expression of fungal inhibtion in LAB analyzed that decreased progressively toward pH 7.0 and failed at alkalin pH. However, sucrose even at high content (8%) was found to have no effect on antifungal activity of LAB, but its bioconversion product glucose and fructose decreased this activity when concentrations were set at 6 et 2 %, respectively. All together, the results suggest that, LAB may effectively exert fungal inhibition in a narrow timing of cocoa fermentation, with pulp contained sugars as metabolic regulators of this inhibition. These results may contribute to a better management of LAB as starter culture for an efficient inhibition of fungal growth and prevention of cocoa contamination from mycotoxins.

Keywords: Antifungal activity; Lactic acid bacteria; Cocoa fermentation; Inhibition variation; pH and temperature; Sugars content

INTRODUCTION

Cocoa is an important crop, as it is the raw material from which chocolate is manufactured (Copetti et al., 2014). Getting chocolate requires a postharvest processing such as fermentation, drying and storage (Camu et al., 2007). However, cocoa beans are susceptible to fungal contamination during these processing steps (mainly drying and storage). These filamentous fungi proliferate during drying and storage when the conditions are favorable for their growth (high humidity, short drying.) (Ruggirello et al., 2019). The presence of filamentous fungi is associated with mycotoxin production in fermented cocoa beans mainly ochratoxin A (Iamanaka et al., 2007; Dembele et al., 2009).

Ochratoxin A (OTA) is a toxic secondary metabolite produced by several species of *Aspergillus* and *Penicillium* genera (Mounjouenpou et al., 2008). OTA attracts particular attention through the damage it does to humans and animals (Abarca et al., 1997). Ochratoxin is linked to adverse health effects such as hepatotoxicity, tetratoxicity and immunosuppression (Bui-Klimke & Wu, 2015). It has also been found to be a carcinogen for the kidney and liver (Pfohl-Leszkowicz & Manderville, 2007). Several studies have revealed the presence of filamentous fungi and ochratoxin A in fermented cocoa beans (Ardhana & Fleet, 2003; Copetti et al., 2014; Copetti et al., 2010; Dembele et al., 2009; Mounjouenpou et al., 2008; Nwagu & Ire, 2011). Therefore, the consumption of chocolate and derived products may be associated with a public health problem due to the presence of ochratoxin A in cocoa. To solve this problem, different approaches are in use to avoid fungal growth and mycotoxins production. These approaches include good post-harvest practices recommending fermentation for 6 six days maximum, drying the fermented beans to decrease humidity below 8 %

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and storing in dry places sheltered from moisture (Copetti et al., 2010). Alongside these agricultural practices to stop OTA production in cocoa, biological methods such as the use of LAB from cocoa fermentation is a promising and potentially efficient approache in addition.

LAB are gaining interest in bio-preservation of food because of the production of antimicrobial compounds such as lactic acid, acetic acid, and bacteriocin (Lavermicocca et al., 2003; Ma et al., 2019; Magnusson et al., 2003; Sayed et al., 2020 Nasrollahzadeh et al., 2022), in addition to be considered as GRAS (Generally Reconized As Safe). The potential of antifungal LAB strains in food quality protection and insurance has been widely and sucessfully reported (Corsetti et al., 1998; Marín et al., 2019; Ruggirello et al., 2019; Sathe et al., 2007; Ayyash et al., 2021). Likewise, LAB were shown to be involved in fungi inhibition during cacao fermentation (Romanens et al., 2019; Rahayu et al., 2021a; Ruggirello et al., 2019; Youte et al., 2020) and their potential to be used as starter to prevent the occurrence of mycotoxin in fermented cocoa and chocolate has been assessed (Marwati et al., 2021; Rahayu et al., 2021; Romanens et al., 2020).

However, it is well assessed that, conditions of cocoa fermentation are dynamic, with changing temperatures from 30 °C to 45 °C, and pH evolving from 3.0 to 6.0 as well as sugars concentrations in the fermenting pulp (De Vuyst & Weckx, 2016; Koffi et al., 2013; Schwan & Wheals, 2004; Soumahoro et al., 2020). In these conditions, the antifungal activity of LAB present in the fermenting cocoa may also be impacted as increased or decreased in response to these changing conditions. Yet this has not been investigated.

The objective of this paper was to analyze the variation of antifungal function of the major LAB species involved in Ivorian cocoa fermentation in relation to changes of culture condition during bacterial growth.

MATERIALS AND METHODS

Biological materials

Fermented and dried cocoa beans used as material were sampled from Agnéby-Tiassa region (geographic coordinates 5°59' North 4°28'West). Thirthy kilograms (30 kg) of beans were randomly collected from farmers and conditioned in stomacher bags then transported to laboratory for microbiological analysis consisting in fungal strains isolation from sampled fermented and dried cocoa beans.

The bacterial material was composed of four different lactic acid bacteria (LAB) strains consisting in 2 *Lactobacillus plantarum* strains (T8N10 and T11G13), a *Lactobacillus casei* strain (T10G5) and a *Leuconostoc mesenteroides* strain (T8AB6) previously isolated from fermenting cocoa in Cote d'Ivoire and identified by 16S gene sequencing, then typed by RFLP; these LAB strains were further characterized at functionnal levels as the dominant LAB strains and potential starter for tunning cocoa fermentation (Ouattara et al., 2017; Ouattara & Niamke, 2021). In the present study, these LAB strains were analyzed for antifungal activity.

Isolation and identification of filamentous fungi from fermenting cocoa beans

Filamentous fungi were isolated on plate agar Sabouraud solid medium. For this purpose, beans were aseptically put on the medium (three beans per petri dish) and incubated at 30 °C for 5 to 7 days. Then the grown fungal colonies around the beans were purified by replication on a new Sabouraud solid medium and the resulting fungies were identified after incubation.

The fungal isolates were identified using cultural and morphological features such as colony growth pattern, conidial morphology and pigmentation as described by Tafinta et al., (2014). Microscopic identification was performed by spreading the mycelium in 10 µL of methylene blue on a slide and the preparation was visualized with a microscope (Geiss, Germany) at × 400 magnitude. The morphological and microscopic characteristics of the fungal isolates observed, allowed their identification basing on the guidelines described by Samson et al. (2007).

Antifungal activity of lactic acid bacteria (LAB)

Antifungal activity of LAB was evaluated in three steps. In the first step, a spore solution was prepared as follow: the isolated fungal strains were grown on agar sabouraud solid medium at 30 °C for 5 days. After incubation, the sabouraud medium containing the cultured fungies was flooded with 10 mL of sterile distilled water to obtain the fungal spore suspension. The concentration of this suspension was estimated by spore count using Malassez cell (Magnusson et al., 2003). In the second step, 0.2 mL of the fungal spore solution was mixed with 10 mL of Sabouraud medium (0.7 % agar) to a final concentration of 10^4 spores/mL, thus a mixed suspension was obtained. In the third step, the mixed suspension was used to overlay a 48h old LAB culture grown on MRS medium in two streaks. The overlaid culture was incubated at 30 °C for 48h, the inhibition zones were measured around the streaks of lactic acid bacteria. The inhibition diameter was measured, and the inhibition rate was determined as a percentage using the following formula as proposed by Magnusson et al. (2003)

$$\text{Inhibition Rate } (iR) = \frac{iD}{D} X\,100$$

iD : inhibition diameter; D : total diameter of the petri dish

Impact of environmental conditions on antifungal activity from LAB

Environmental conditions retained for this study are those susceptible to influence LAB growth and their antifungal activities. These conditions are essentially, pH, temperature and sugars concentrations as the mains culture conditions prevailing in natural cocoa fermentation (Ouattara & Niamke, 2021). The impact of these conditions on antifugal activity was measured by varying the culture pH from 3 to 8, the range of temperatures from 30 to 45 °C and sugars concentration notably glucose, fructose and sucrose from 2 to 8 %. The antifungal activity were evaluated by overlay assay as described above.

HPLC essay of acids produced from antifungal LAB strains

The assay of acids production from LAB was conducted as we previously described (Ouattara et al., 2017). The bacteria were grown in MRS liquid medium for 48h at 30 °C. Then, after centrifugation for 10 min at 12,000 × g, cells free supernatant were collected and applied on a HPLC system equipped with a column Hypersyl GOLD aQ (250 × 4.6 mm), with a particle size of 5 µm (Thermo Fisher, France). To achieve a separation with high resolution, the elution solvent A was composed of 50 mM KH_2PO_4 MilliQ water made with pH adjusted to 2.65 with H_2PO_4 and the solvent B was acetonitrile (or methanol), all of HPLC grade. Separation was performed at 25 °C with a flow rate of 1 mL/min and under the following conditions: pre-equilibration for 10 min in buffer A before injection; 0 min, 100% A; 8 min, 100% A; 10 min, 100% B; 12 min, 100% B; 13 min 100% A; 15 min, 100% A followed by a new injection, as described. The detection of the lactic, acetic and citric acids peaks was performed at a wavelength of 210 nm and a spectrum from 210 to 400 nm. Identification of compounds in the cells free supernatant was achieved by co-injection with authentic standards (Sigma-Aldrich, France) and peaks comparison. Under our conditions, lactic, acetic and citric acids were eluted at retention times of 4.70, 5 and 6.10 min, respectively. Quantification was performed using standard curve obtained by increasing the injected quantity of each acid to determine the slope as a correcting factor.

Statistical analysis

All the experiments were done in triplicate, and the results were presented as mean values ± standard deviation. When required, an analysis of variance (ANOVA) was performed with the software SPSS version 20.0®, using Duncan test at 95 % confidence to check significant differences between values.

RESULTS

Isolated and identified filamentous fungi from cocoa

From a random set of sample including 30 fermented and dried cocoa beans, a total of 68 colonies were found on the surface of the sabouraud media directly seeded with the beans. Then, macroscopic and microscopic observations (described above) allowed the clustering of the 68 fungal colonies into six groups, each group of colonies sharing the same features as the same isolate (Fig. 1). Among the six isolates, 2 presented features caracteristic of the genus *Mucor* while the 4 other isolates showed caracteristics presomptive of *Aspergillus* (Fig. 1). Aspergillus isolates were characterized by velvety colony initially white but turned into yellow, green or black toward the end of the culture, depending on the type of colony. At the microscopic observation, the mycelium of Aspergilli were septate with unique head of conidiophore (not divided). Colonies of Mucor were white or brown at the end of the culture depending on the type of colony, the mycelium was branched, but not septate.

Different levels of antifungal activity from various lactic acid bacteria

The different well characterized LAB strains from fermenting cocoa were then analyzed for their antifungal capacities, using the isolated fungies as target. The results showed that LAB were able to inhibit both conidial germination and mycelial growth of the six isolated filamentous fungi (Fig. 2). To further evaluate and quantify the antifungal capacities of the various LAB strains, the inhibition rate was calculated using a direct contact LAB-Fungies approach by the overlay method (described in material and method section). The results show different inhibition levels depending on the LAB and fungal strains (Fig. 3 and Fig. 4).

A total inhibition (100 % inhibition) of the two *Mucor sp* isolates, was achieved with all LAB strains (Fig. 4). Likewise, a strong inhibition of the *Aspergillus* isolate Asp 04 was also reached with all LAB strains recording more than 89 % of inhibition rate. However, the *Aspergillus* isolate Asp 01, proved to be the less susceptible to LAB antifungal activities that were relatively lower against this fungal isolate. Regarding the inhibition rates, two groups of fungal isolates were observed: a group of fungal isolates that were completly inhibited by the LAB strains (more than 90 % inhibition) notably Muc01, Muc02 and Asp 04, and the second group including fungal isolates that could not be completly inhibited (less than 80 % inhibition) namely Asp 01, Asp 02 and Asp 03 (Fig. 4). Basing on the inhibition rate obtained on the second fungal group (not completly inhibited), the LAB strain *Leuconostoc mesenteroides* T8AB6 produced the weakest antifungal activity whereas the *Lactobacillus plantarum* strains T8N10 proved to be the most efficient on fungies (Fig. 4).

Relationship between acids production and antifungal activity in LAB

Acids are known to presumably be the first compounds that may impart to LAB, their antimicrobial properties. In

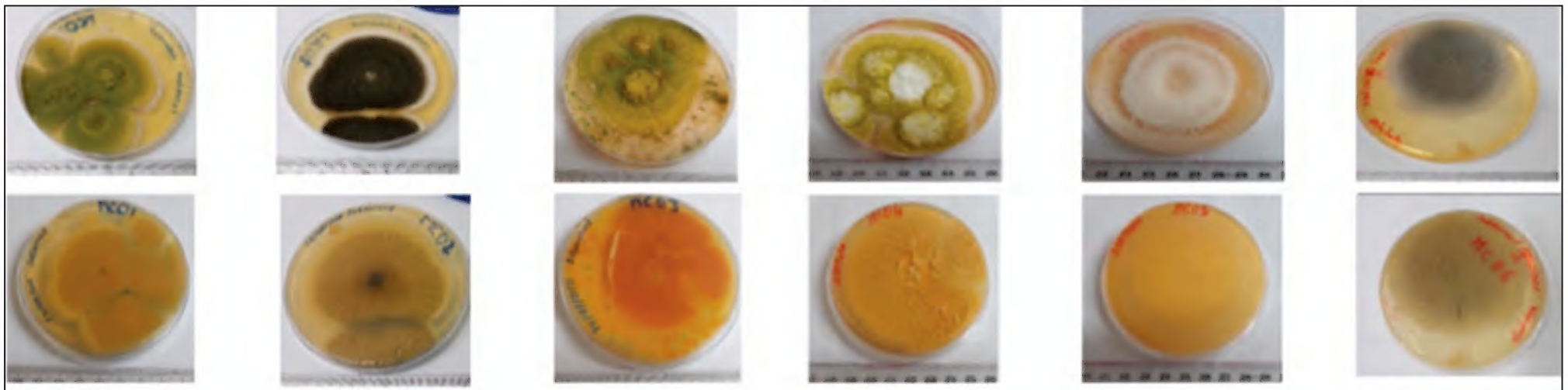
Aspergillus sp 01
(13 isolates)
On the front : Colony powdery green-yellow
On the reverse side : colony brown-red
Aspergillus sp 02
(20 isolates)
On the face: flat, fluffy colony with white mycelium
and black spores
On the reverse side: pale yellow colony
Aspergillus sp 03
(11 isolates)
On the front: powdery, green to yellow colony
On the reverse: reddish colony
Aspergillus sp 04
(12 isolates)
On the front: white-yellow colony
On the reverse: colourless colony
Mucor sp 01
(04 isolates)
On the face: whitish woolly colony
on the reverse: colourless colony
Mucor sp 02
(08 isolates)
On the face: woolly colony with black mycelium
and white spores
On the reverse: colourless colony

Fig 1. Diversity of fungi isolated from fermented and dried beans. Fungal isolates were obtained on sabouraud medium after 7 days culture at 30 °C.

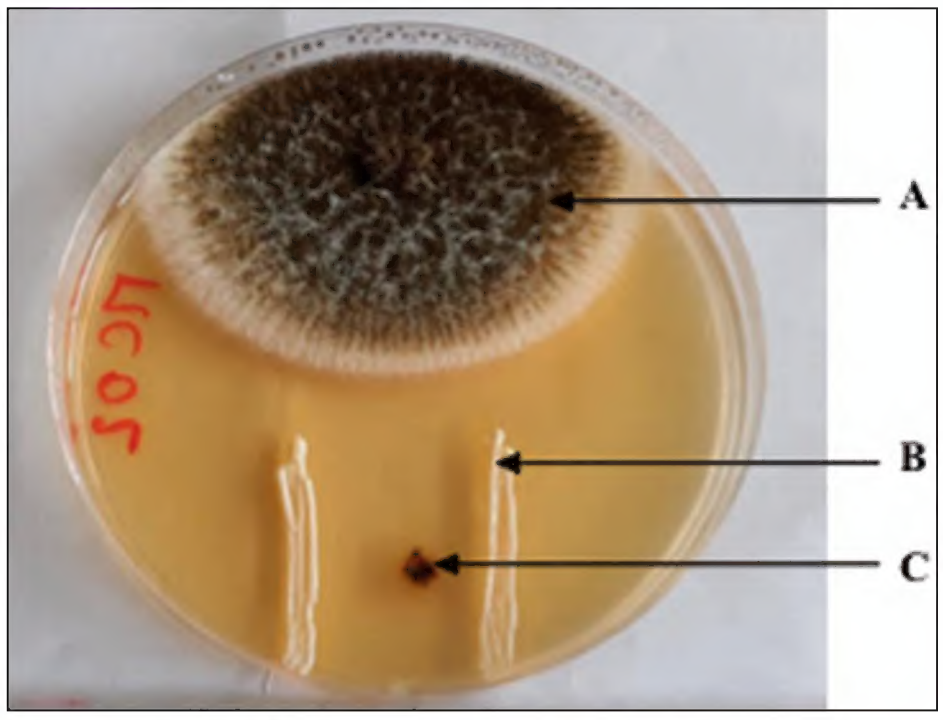

Fig 2. Evidence of fungal inhibition by LAB strains. A Fungal colony developed out of LAB area; B Streak of LAB strain after 48h growth; C Fungal spore unable to grow near the LAB streaks.

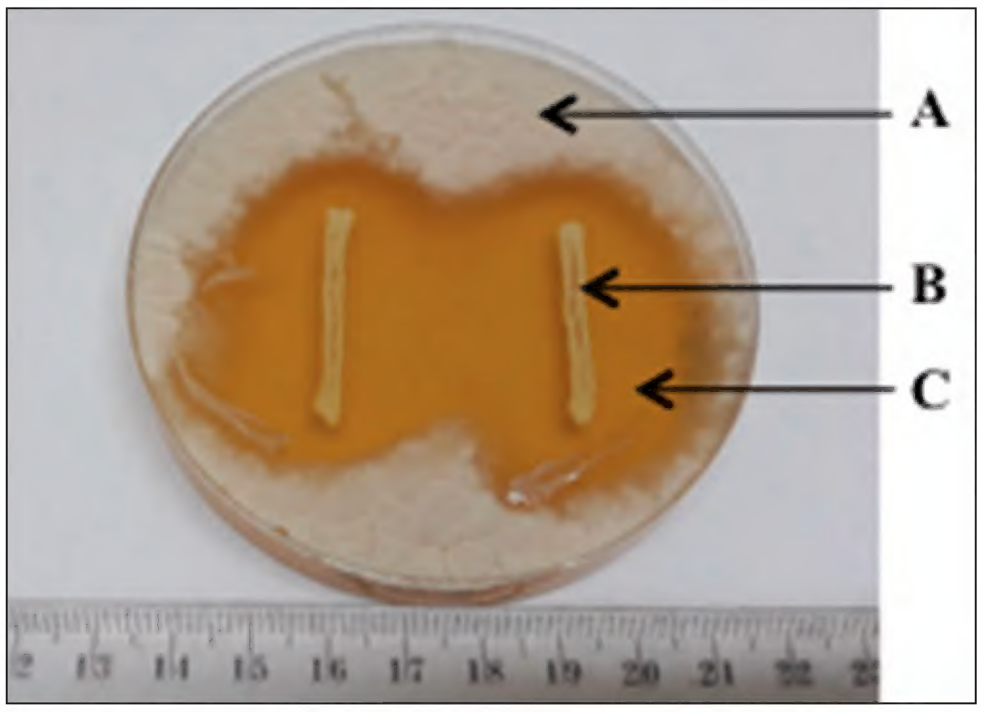

Fig 3. Antifungal activity of *Lactobacillus plantarum* T11G13 assayed by overlay method. (A) strong fungal growth ; (B) streak of lactic acid bacteria. (C) area of inhibition with no fungal growth around the streak of lactic bacteria. The inhibition rate (iR) was calculated as a percentage (%) using the following formula : iR = (iD/D) .100 ; where iD is the diameter of inhibition area and D is the total diameter of petri dish. Magnusson et al., 2003.

this section, we investigated whether acid production may take an important role in the antifungal activity observed in LAB strains analyzed.

The HPLC assay showed that LAB studied produced mainly lactic acid and secondarly acetic acid. The production of lactic acid was at least 10 folds more important than acetic acid (Table 1). *Leuconostoc mesenteroides* yielded the highest quantity of total acid with 5.32 mg/ml while the other strains *Lactobacillus casei* (T10G5), *Lactobacillus plantarum* strain T11G13 and *Lactobacillus plantarum* strain T11G13 showed approximately the same acid production 3.16 to 3.49 mg/mL (Table 1). Although these strains yielded roughly the same quantities of acids, their antifungal activity were different against Asp 02 and Asp 03 (Table 1). Moreover, *Leuconostoc mesentoroides* as the best acid producer presented the lower antifungal activity against Asp 03 and Asp 02. The production of acids compound by LAB may not be correlated with their antifungal activity.

Effects of temperature, and pH on the antifungal activity in LAB strains

Among the different fungal isolates, we have observed that Asp_02 showed caracteristics close to *Aspergillus niger*, an ochratoxin producing species, notably large head of conidiophore with black color due to darkly pigmented conidia Fig 1, (Toma et al., 2021). This isolate (Asp_02) was therefore used as model in further studies, notably the influence of culture conditions on the antifungal activity of LAB strains involved in ivorian cocoa fermentation.

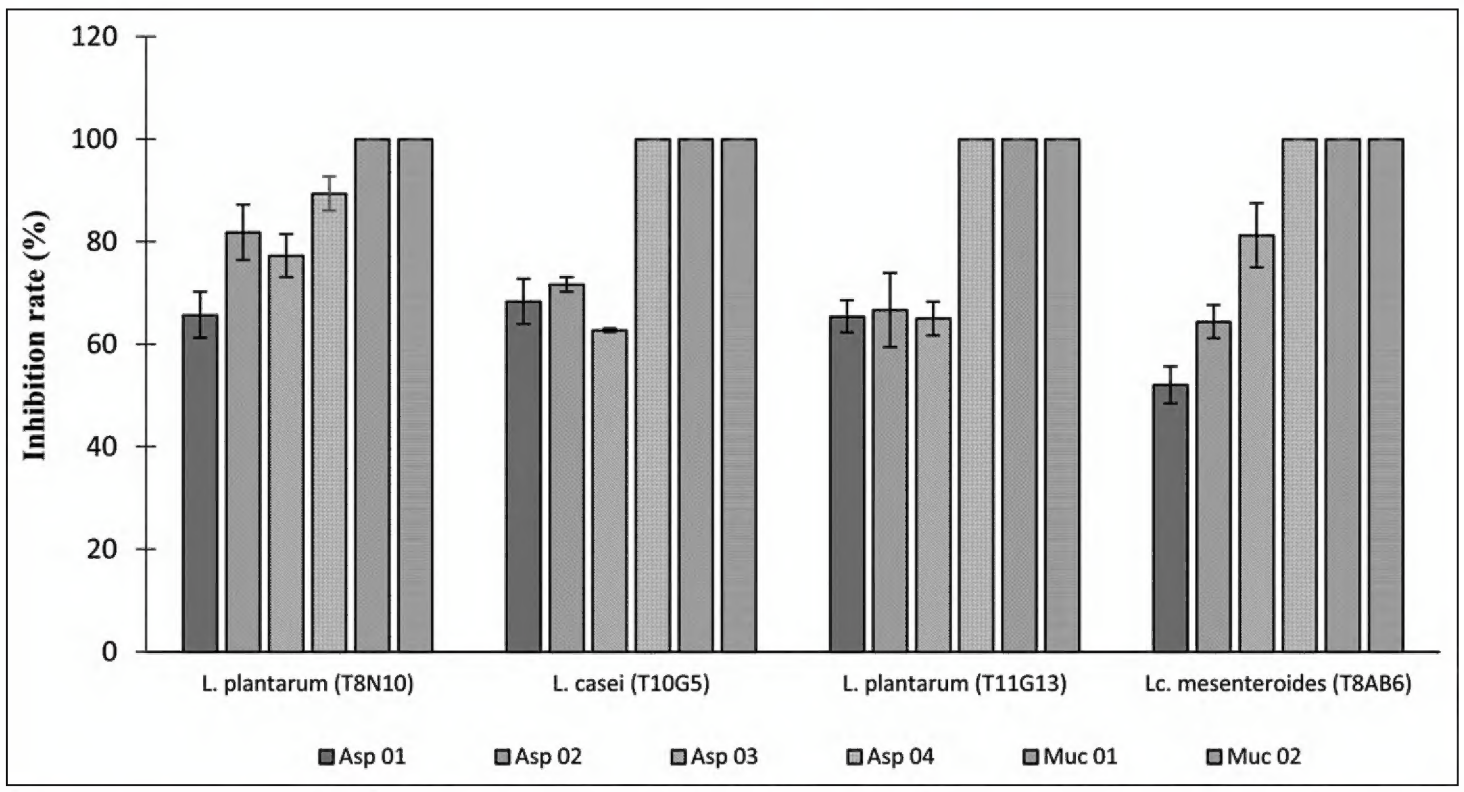

Fig 4. Inhibition rate of lactic acid bacteria strains against various fungal isolates. MRS medium was used for LAB growth and their antifungal assay against fungal strains Incubation conditions were 30 °C for 48h.

Effect of temperature

All the LAB strains showed maximum antifungal activity in the range of 30-35 °C as they were able to express at least 80 % of their antifungal potential at these temperatures (Fig. 5). *Lactobacillus plantarum* strain T8N10 and *Leuconostoc mesenteroides* T8AB6 exerted the maximum antifungal activities (100 % of their potential) at 35 °C, whiles the maximum antifungal action of *Lactobacillus casei* strain T10G5 and *Lactobacillus plantarum* strain T11G13 occured at 30 °C. Out of this range at 40 °C, a notable decrease of antifungal action of LAB was observed but the strains remained able to express roughly 50 to 60 % of their potential. However, this decrease of antifungal capacities at 40 °C, was particularly severe in the *Lactobacillus casei* strain T10G5 that kept only 22.58 % of its potential. At 45 °C, the antagonistic action of LAB on fungies was lower, expressing less than 20 % in *Lactobacillus plantarum* strain T8N10 and *Lactobacillus casei* strain T10G5 and less than 40 % in *Lactobacillus plantarum* strain T11G13 and *Leuconostoc mesenteroides* T8AB6 (Fig. 5).

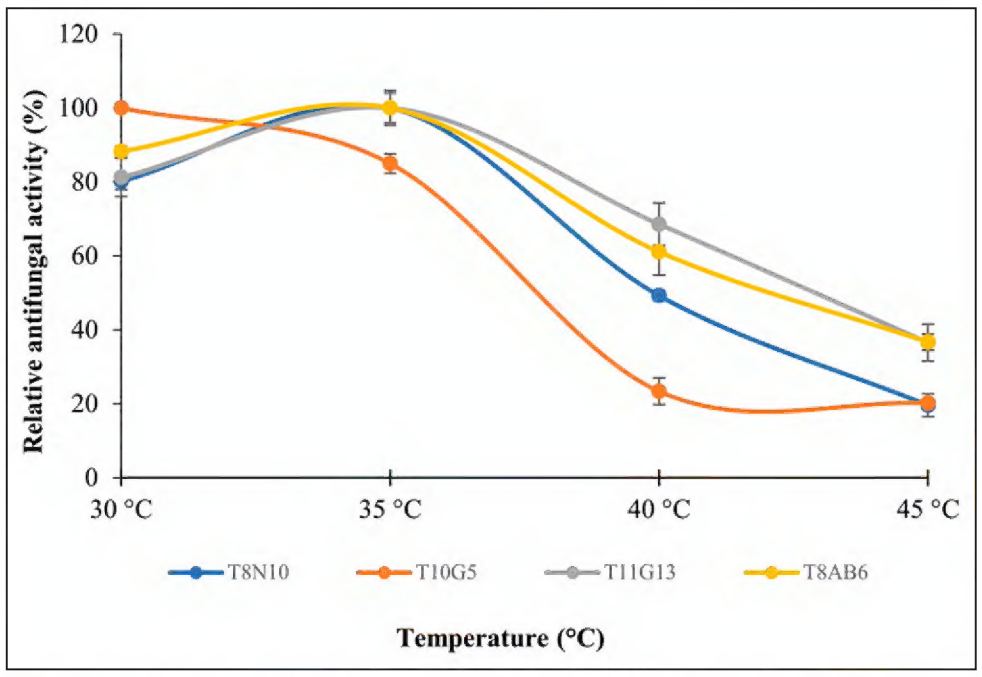

Fig 5. Variation of antifungal activity of LAB strains cultured under different temperatures conditions. MRS medium was used for LAB growth and their antifungal assay against fungal strains. Incubation was performed at various temperatures for 48h.

Effect of pH

The action of LAB strains against fungal growth was stronger in the range of acidic pH, between 3 and 5, where more than 80 % of their antifungal potential was expressed (Fig. 6). However, exception was observed with *Lactobacillus plantarum* strain T11G13 that was efficient against fungies in a narrower pH range pH 3-4, producing only 32 % of antifungal potential at pH 5. When pH was set at 6, all LAB strains lost at least 50 % of their antifungal activities except *Leuconostoc mesenteroides* T8AB6 that still kept 78 % of antifungal activity. Even at pH 7, *Leuconostoc mesenteroides* T8AB6 expressed 56 % of activity against fungies. Towards neutral pH, all antifungal activies from LAB continously decreased, then these bacteria completly failed to produce antifungal activity at alkalin pH (Fig. 6).

Antifungal activities of LAB strains, as affected by carbon metabolism

The influence of sugar concentration on the antifungal activity of lactic acid bacteria is shown in Table 2. Increasing glucose concentrations to 6 % in the culture medium, allowed roughly 1.5 folds increase of the antifungal activity of LAB strains notably in *Lactobacillus plantarum* strain T8N10 and *Lactobacillus casei* strain T10G5. However, this increase of antifungal activity was not notable with the strain *Lactobacillus plantarum* T11G13. Particular attention was payed with the LAB strain *Leuconostoc mesenteroides* T8AB6 since not only the range of glucose concentration in which, this strain could produce

Table 1: Antifungal activity and acid production in LAB bacterial strains

LAB strains	Antifungal inhibition rate (%)			Acids production (mg/mL)		
	Against Asp_01	Against Asp_02	Against Asp_03	Lactic	Acetic	Total Acids
L. plantarum (T8N10)	65.71±4.81^{a}	81.81±4.58^{a}	77.29±7.01^{a}	2.82±0.48^{a}	0.34±0.04^{a}	3.16±0.82^{b}
L. casei (T10G5)	68.33±2.33^{b}	71.67±6.79^{b}	62.71±3.19^{b}	3.17±0.77^{b}	0.32±0.05^{a}	3.49±0.50^{b}
L. plantarum (T11G13)	65.42 5.47^{a}	66.67±3.29^{c}	65.00±3.57^{b}	3.01±0.64^{b}	0.48±0.10^{b}	3.49±0.64^{b}
Lc. mesenteroides (T8AB6)	52.08±3.15^{c}	64.38±2.30^{c}	81.25±5.2^{a}	5.08±1.20^{c}	0.24±0.09^{c}	5.32±1.07^{a}

MRS medium was used for LAB growth and their antifungal assay against fungal strains. Incubation conditions were 30°C for 48h.
Data are represented as means±SEM (n=3). Mean with different letters in the same colunm of acid production and fungal inhibition rate are statistically different ($p<0.05$) according to Duncan's test

Table 2: Effect of sugars concentrations on antifungal activity of LAB bacterial strains

		L. plantarum (T8N10)	*L. casei* (T10G5)	*L. plantarum* (T11G13)	*Lc. mesenteroides* (T8AB6)
% Glucose	2	69.98±5.1^{b}	69.18±6.3^{bc}	80.13±3.8^{b}	82.24±4.5^{b}
	4	74.71±3.8^{b}	83.02±5.7^{b}	100.0±3.6^{a}	100.0±6.3^{a}
	6	100.0±2.2^{a}	100.0±3.6^{a}	96.50±5.2^{a}	20.07±2.2
	8	40.13±4.4^{c}	59.22±3.1^{c}	41.05±0.0^{c}	18.47±1.9^{c}
% Sucrose	2	100.0±3.8^{a}	94.76±3.1^{a}	88.2±2.6^{ab}	67.91±3.1^{b}
	4	90.09±5^{ab}	100.0±2.6^{a}	93.45±4.3^{a}	85.55±4.4^{ab}
	6	76.57±0.7^{b}	73.80±1.9^{b}	72.58±1.4^{b}	100.0±3.1^{a}
	8	100.0±4.4^{a}	82.97±1.0^{ab}	100.0±1.4^{a}	97.68±2.5^{a}
% Fructose	2	100.0±2.0^{a}	100.0±1.9^{a}	100.0±0.0^{a}	100.0±2.4^{a}
	4	54.62±3.1^{b}	81.86±2.6^{ab}	50.00±0.0^{b}	47.02±1.9^{b}
	6	52.24±2.5^{b}	78.43±1.3^{b}	28.40±1.4^{c}	57.96±3.3^{b}

Note: Data are represented as means±SEM (n=3). Mean with different letters in the same colunm are statistically different ($p<0.05$) according to Duncan's test. The fungal isolate *Aspergillus sp* was used as the model target

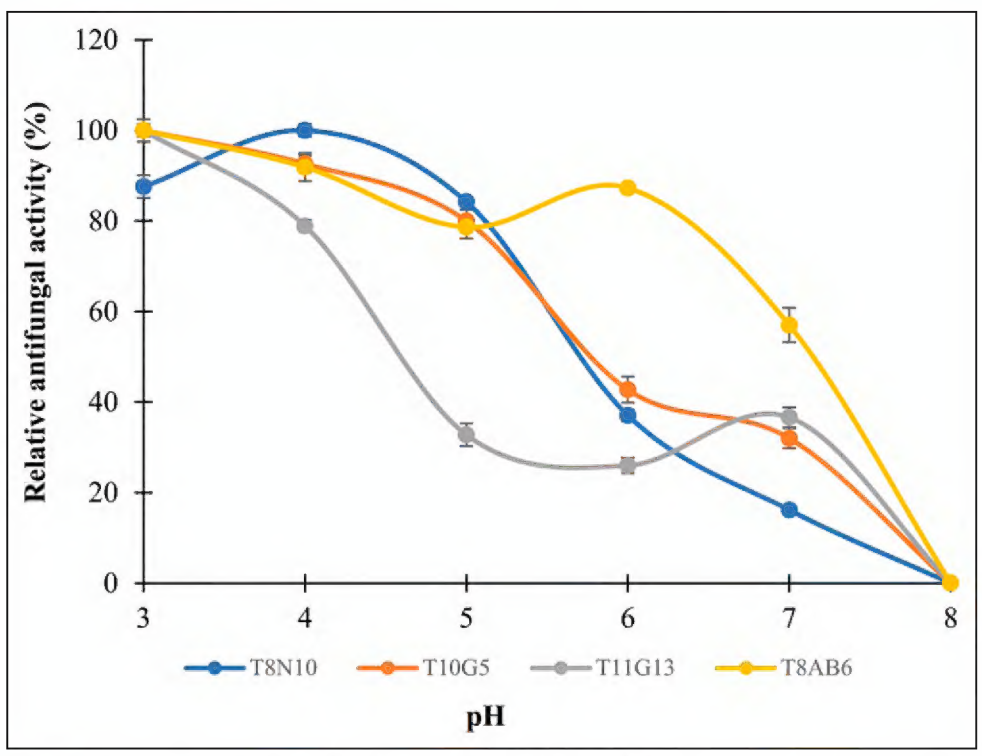

Fig 6. Variation of antifungal activity of LAB strains cultured under different pH conditions. MRS medium was used for LAB growth and their antifungal assay against fungal strains. Incubation conditions were 30 °C for 48h at various pH.

antifungal activity was relatively narrow (only 2 to 4 % of glucose) but also, out of this range, it was observed a sharp decrease of antifungal activity. Likewise, the other LAB strains decreased their antifungal activity above a glucose concentration of 6 %.

When fructose was used as carbon source, the maximum antifungal activity was achieved at relatively low fructose concentration, notably at 2 %. Then increasing fructose content lead to a progressive decrease of LAB's antifungal activities in all strains. These strains failed to express their antifungal activity at fructose concentration of 8 %.

In contrast, sucrose was found to have less effect on the antifungal activity of LAB strains since no notable variation was observed in the range of sucrose concentration studied (2 to 8 % of sucrose) (Table 2).

DISCUSSION

In this study, we have evaluated the antifungal function of the main LAB strains involved in Ivorian cocoa fermentation, and analyzed their potential to express this function under different culture conditions.

In the first step, fungies were isolated from cocoa. The obtention of 68 fungal isolates essentially Aspergillii and Mucors from a small set of fermented and dried cocoa sample show that cocoa is subjected to fungal contamination perhaps at unsuspected high level, although the fungal diversity isolated here was limited. Many studies have reported the presence of molds during fermentation drying and storage (Ardhana & Fleet, 2003; Copetti et al., 2014; Schwan & Wheals, 2004; Guehi et al., 2007) with a predominance of the *Aspergillus sp*. And/or *Mucor sp*. in accordance with the fungal species found in this work. However, few studies also indicated the presence of *Penicillum sp*. in addition to *Aspergillus sp*. and *Mucor sp*. in cocoa (Sánchez-Hervás et al., 2008). Molds in cocoa

are generally associated with off flavours, high level of free fatty acids and production of mycotoxins (Delgado-Ospina et al., 2021; Hansen, Welty et al., 1973; Maciel et al., 2018), impairing the quality of cocoa beans and raising the problem of health risk for consumers (Romanens et al., 2019). Therefore, the control of fungal growth during farm processing of cocoa is of high importance to ensure quality cocoa and chocolate.

The analysis of antifungal activity of the majors LAB strains notably *Lactobacillus plantarum* strains (T8N10 and T11G13), *Lactobacillus casei* strain (T10G5) and a *Leuconostoc mesenteroides* strain (T8AB6) involved in ivorian cocoa fermentation showed that all the LAB strains were able to inhibit fungal growth, *Lactobacillus plantarum* T8N10 being the best inhibitor of the targeted fungi. Generally, *Lactobacillus plantarum* as one of the most dominant LAB involved in cocoa fermentation worlwide (De Vuyst & Weckx, 2016; Lefeber et al., 2011; Ordoñez-Araque et al., 2020; Ouattara et al., 2017; Ouattara & Niamke, 2021) are the most reported LAB species having antifungal activity (Rahayu et al., 2021; Ruggirello et al., 2019; Youte et al., 2020) together with *Lactobacillus fermentum* (Romanens et al., 2020). In contrast, *Leuconostoc mesentoides* displaying the weakest antifungal activity in this work, was not previously known as inhibiting fungal growth in cocoa fermentation.

Moreover, the different levels of fungal growth inhibition observed from the LAB species may be due to probable difference in the active compounds produced, but also in the difference of regulation of these actives compounds with regards to the cultures conditions notably pH and temperature. Indeed, fungal inhibition by LAB strains was found in this study to be impacted by the different culture conditions. Maximum fungal inhibition occured in the range 30-35° C while active pH ranged from 3.0 to 4.0. Similar observations were made by Magnusson & Schnürer, (2001) reporting that, maximum antifungal activity in *Lactobacillus coryniformis subsp. coryniformis* strain Si3, occured at pH between 3.0 and 4.5, but it decreased rapidly when pH was adjusted to a level between 4.5 and 6.0 and was lost at higher pH values.

The temperature and pH conditions at which maximum antifungal activity was produced by LAB strains in the present study exactly match with the maximum growth of LAB during cocoa fermentation, where LAB peak genearally after 36-48h of fermentation in the condition of pH 3.5-4.5 and temperature around 35° C (Ouattara & Niamke, 2021; Schwan & Wheals, 2004; Soumahoro et al., 2020). The studied LAB strains may effectively produce antifungal activity during cocoa fermentation. Indeed Rahayu et al. (2021) recently reported that LAB strain among other microorganisms can inhibit fungal growth and mycotoxin production during cocoa fermentation.

However, our study also showed that high glucose and fructose contents decrease the fungal inhibition above a concentration of 6 % and 2 %, respectively. Likewise, glucose was previously found to decreased the activity of antifungal agents against candida spp. (Mandal et al., 2014) suggesting that sugars may not reduce the synthesis of antifungal agent in LAB, but rather may inhibit the action of the synthesized antifungal agent.

On the other hand, it was observed in this study that, sucrose has no effect, even at high concentration (8 %) on antifungal activity of LAB. Delavenne et al. (2015) also found that *Lactobacillus harbinensis* maintained a stable antifungal activity over time, which was not affected by initial sucrose concentration. These results suggest a metabolic regulation of antifungal activity in LAB strains by the sugars content during fermentation. The bioconversion of the high sucrose content contained in the pulp into glucose and fructose may be a key factor modulating LAB's antifungal activity in fermenting cocoa.

In this study, we also investigated whether acids production take an important place in the observed antifungal activity of the analyzed LAB. The results showed no obvious correlation between acid production and the strengh of fungi inhibition, suggesting that compounds other than acids may be involved in the antifungal inhibtion by LAB. Likewise, Magnusson et al. (2003), also reported that the degree of fungal inhibition in an environmental isolate of *Lactobacillus plantarum* was not only related to production of lactic acid or acetic acid, but also to an antifungal cyclic dipeptides. Moreover, bioactive antifungal compounds from *Lactobacillus harbinensis* Kv931 were identified by nuclear magnetic resonnance and Mass spectroscopie as a mixture of benzoic acid and a polyamine (Mosbah et al., 2018), whereas the responsible antifungal compounds from *Leuconostoc mesenteroides* were identified as a mixture of lactic acid, acetic acid and an unidentified hydrophobic molecule (Lee & Chang, 2016). Hence, the diversity of LAB strains may comtribute to a diversity of active antifungal compound with a possible synergistic action that may lead to an efficient inhibition of the fungal growth in cocoa fermentation. Th nature of active compounds produced by studied LAB strains will be further insvestigated.

CONCLUSION

This study showed clearly that, cocoa fermentation related conditions (temperature, pH, sugar content) exert *in vitro* a notable influence on fungal inhibition by LAB.

This influence is characterized by a maximum fungal inhibition occuring in a narrow pH and temperature ranges (temperature 30-35 °C and pH 3.0-4.0) and sugars concentrations less than 2 %. With regards to natural cocoa fermentation that always begin in acidic pH (3.0-4.0), lower temperature (30-35 °C), LAB may potentially express their antifungal activity during the first stage. Taking into account these conditions may help to better manage the use of LAB strains as starter culture to efficiently inhibit fungal growth and prevent cocoa contamination from the mycotoxin OTA.

Authors' contributions

Honore G. Ouattara designed the research. Honoré G. Ouattara and Wilfried Yao planned the work with Karou Germain and Niamké Sebastien as supervisors. Wilfried Yao and Hadja D. Ouattara were involved in the implementation of the research. All authors discussed the results and commented on the manuscript.

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